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Comparative study of polyethylenimines for transient gene expression in mammalian HEK293 and CHO cells

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ABSTRACT

Three commercially available linear polyethylenimines (25 kDa LPEI, 40 kDa PEI"Max" and PEIpro™) were compared regarding their potency to transfect serum-free growing and suspension-adapted HEK293 and CHO cells. We determined the optimal DNA:PEI ratios for maximal expression of the reporter gene SEAP while monitoring cytotoxicity following transfection. PEIs acylation was determined by ^{1}H NMR and their apparent size and polydispersity assessed by size-exclusion chromatography. The propensity of PEIs to condense plasmid DNA was evaluated by agarose-gel electrophoresis. The zeta potentials and particle sizes at optimal DNA:PEI ratio were analyzed. Polyplex attachment to the cells and internalization kinetics were monitored. The quantity of PEIpro™ needed to efficiently transfect the cells was significantly lower than with LPEI and PEI"Max" and, interestingly, the maximal amount of internalized PEIpro™-based polyplexes was approximately half of that observed with its counterparts. PEIpro™ was the largest and least polydisperse polymer, but also the most cytotoxic. The optimal transfection conditions were subsequently used to express three monoclonal antibodies at larger-scale. The use of the deacylated PEI"Max" and PEIpro™ resulted in a significant increase of recombinant protein expression compared to LPEI. These findings demonstrate the importance of properly choosing the most suitable polymers to obtain optimal recombinant protein transient expression.

1. Introduction

Large-scale transient gene expression (TGE) in mammalian cells is widely used for the fast production of recombinant proteins (r-proteins) that require proper folding, assembly and post-translational modifications for their biological activities. TGE can generate milligram to gram quantities of r-proteins within a few days (Baldi et al., 2007; Geisse and Fux, 2009; Pham et al., 2006) and thus supersedes the long, tedious and costly process associated with the establishment of high-producing stable cell lines. CHO (Chinese hamster ovary) and HEK293 (human embryonic kidney 293) cell lines have been successfully used as transient expression systems. While transfection of serum-free adapted and suspension-growing mammalian cells can be performed by a variety of transfection reagents (Chahal et al., 2011), polyethylenimine (PEI), a cationic polymer, is probably the most widely used, characterized and cost-effective reagent for large-scale applications (Geisse and Fux, 2009; Pham et al., 2006).

Linear PEI (LPEI) with an average molecular weight of 25 kDa is routinely used for TGE because it exhibits high transfection efficiencies and low cytotoxicity (Aravindan et al., 2009; Dai et al., 2011; Nimesh et al., 2007). LPEI contains residual N-propionyl groups that reduce the number of protonatable nitrogen residues and as such may limit nucleic acid condensation and endosomal escape (Thomas and Klibanov, 2002). Indeed, nearly fully deacylated LPEIs have been developed and shown to be superior for mammalian cell transfection (Jeong et al., 2001; Thomas et al., 2005). Commercially available deacylated PEIs are known as PEI"Max" (Polysciences) and PEIpro™ (Polyplus Transfection). The impact of LPEI acylation levels on transfection efficacy has however shown contradictory results (Forrest et al., 2004; Gabrielson and Pack, 2006; Kadlecova et al., 2012).

Improvement of large-scale mammalian cell transfection systems can be achieved through fine-tuning multiple parameters such as the expression vector, transfection reagent, cell line, culture medium and feed addition post-transfection. The proper selection of the transfection reagent thus represents an easy parameter to optimize and may have significant impact on TGE process. With

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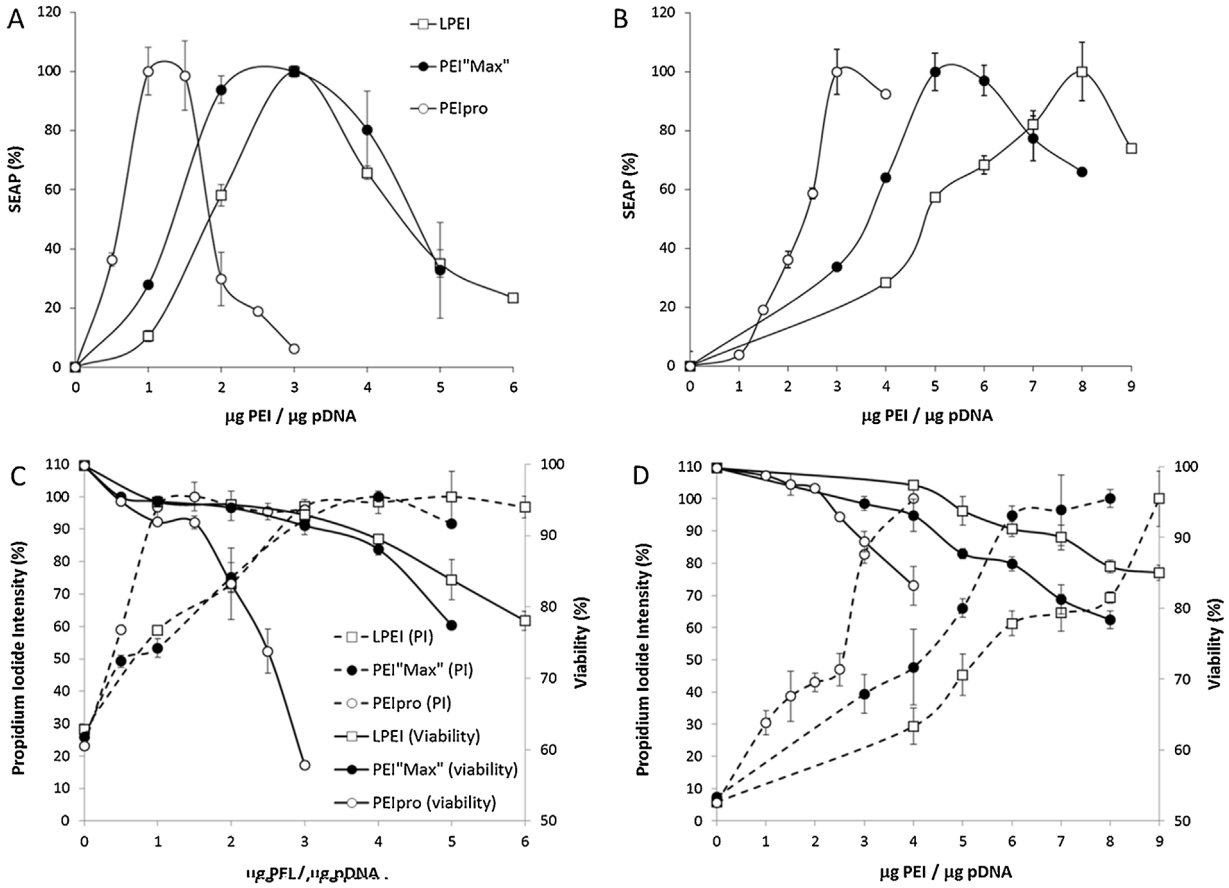

Fig. 1. PEIs-mediated transfection of HEK293 and CHO cells, propidium iodide labeling and viability determination. Increasing concentrations of polymers were used to form polyplexes using 1 µg of plasmid DNA. The SEAP activity present in the culture medium from HEK293 (A) and CHO (B) cells was determined at day 6 post-transfection and reported as percentage of maximal SEAP expression for each polymer. Propidium iodide labeling was used to measure the membrane integrity of HEK293 (C) and CHO (D) cells 48 hpt. Viability (as determined by flow cytometry analyses) of transfected HEK293 (C) and CHO (D) cells was determined at 48 hpt. Results were normalized to controls without transfection and are representative of three independent experiments conducted in triplicate.

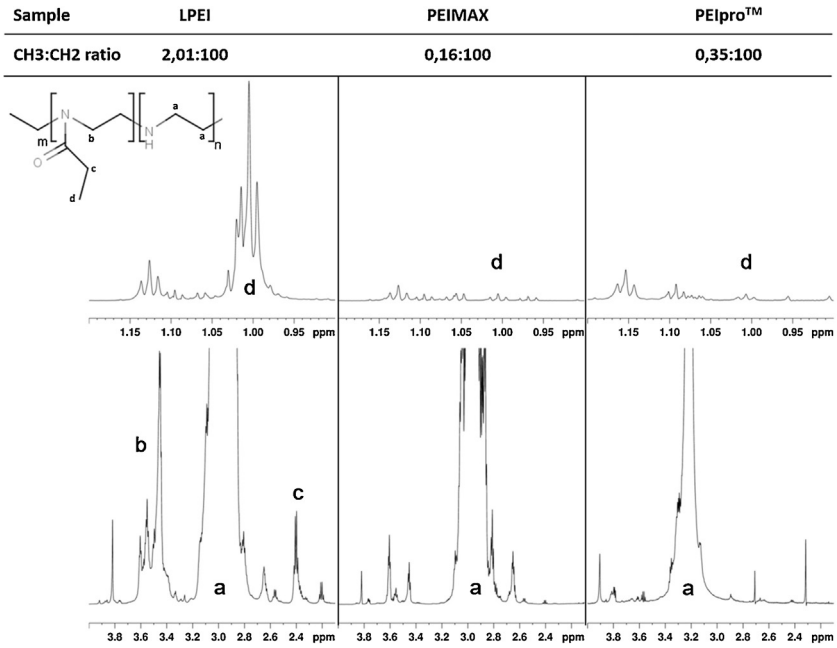

Fig. 2. 1D ^{1}H NMR spectra of partially acylated LPEI and fully deacylated PEI"Max" and PEIpro™. a and b correspond to CH_2 residues in the PEI backbone while c and d correspond respectively to the CH_2 and CH_3 residues in the propionyl side-chain.

Table 1
Vectors used in this study. Expression of the genes of interest are under the control of the constitutive promoter CMV5 (Massie et al., 1998).

Vector name	Description
pTT-BFP	blue fluorescent protein
pTToc3-SEAP	secreted alkaline phosphatase
pTT5-B723LC	B72-3 light chain
pTT5-B723HC	B72-3 heavy chain
pTT5-SNGSLC	Synagis light chain
pTT5-SNGSLH	Synagis heavy chain
pTT5-TZMLC	Trastuzumab light chain
pTT5-TZMHC	Trastuzumab heavy chain

this aim in mind and to further understand the impact of structure-function relationship of PEI on TGE bioprocesses, we characterized and compared the potency of three commercially available PEIs, namely the 25 kDa LPEI, 40 kDa PEI"Max" and PEIpro™ for their abilities to transfect HEK293 and CHO cells growing in suspension culture in the same serum-free medium. ^{1}H NMR analysis of the three PEIs revealed that the degree of acylation is very low (≤3%) in all three polymers, with PEI"Max" being the most deacylated one. Determination of the apparent molecular weight and polydispersity by size-exclusion chromatography showed that LPEI and PEI "Max" are very polydisperse while PEIpro™ is significantly larger and monodisperse. All polymers showed similar capacity to condense plasmid DNA as shown by agarose-gel electrophoresis. Under optimal DNA:PEI ratio for achieving maximal r-protein expression, the polyplexes average size and zeta potential at physiological pH were relatively similar. Correlation between the r-protein yield and the polyplexes uptake and endocytosis kinetics as well as cytotoxicity post-transfection was also investigated. The optimal transfection conditions determined at small-scale were then applied to express three monoclonal antibodies (mAbs) in HEK293 and CHO cells at the 500 mL-scale in shaker flask cultures. The results confirmed that PEI"Max" and PEIpro™ were the most efficient polymers for achieving highest productivities in the two cell lines.

2. Materials and methods

2.1. Materials

The 25 kDa LPEI and 40 kDa PEI"Max" were obtained from Polysciences (Warrington, Pennsylvania, USA). PEIpro™ was from Polyplus-transfection (Illkirch, France). YOYO-1® iodide (1 mM solution in DMSO), trypan blue solution 0.4% (w/v), serum-free Freestyle™ 17 (F17) expression medium and propidium iodide (PI) were purchased from Life Technologies Inc. (Burlington, Ontario, CA). The pTT expression vectors (Durocher et al., 2002) encoding for the blue fluorescent protein (BFP), secreted alkaline phosphatase (SEAP), *Trastuzumab*, *Palivizumab*, *or chimeric B72-3* (Xiang et al., 1990) ($h\gamma_4$) light (LC) and heavy (HC) chains were used to optimize and monitor the transfection process (list in Table 1). Plasmids were amplified in *Escherichia coli* (DH5α) and purified using an in-house endotoxin-free process (unpublished). L-glutamine and Pluronic® F-68 were obtained from Sigma-Aldrich® (Oakville, Ontario, CA).

2.2. Cell culture

The 293-6E and CHO-3E7 cell lines stably expressing truncated Epstein-Barr virus Nuclear Antigen-1 were maintained as a suspension culture in F17 medium supplemented with 4 mM L-glutamine and 0.1% pluronic® F-68. CHO-3E7 cells were routinely diluted every two to three days at 0.3×10^6 cells/mL (0.07×10^6 cells/mL for weekends) in 50 mL total volume in a 250 mL plastic Erlenmeyer flask (Corning®). 293-6E cells were also diluted every two to three days at 0.5×10^6 cells/mL (0.25×10^6 cells/mL for weekends). Cell cultures were operated on an orbital shaking platform at an agitation rate of 120 rpm at 37 °C in a humidified 5% CO_2 atmosphere. Cell density and cell viability were determined by direct counting of cell samples with a Cedex automated cell counting system (Roche Diagnostics, Laval, QC, Canada) using the trypan blue exclusion method.

2.3. Transfection

Transfections were performed at a cell density of 2.0×10^6 cells/mL with viability greater than 98%. The cell suspension was distributed in 6-well plates (1.8 mL/well). Prior to transfection and when mentioned, the pTT-BFP reporter plasmid was labeled by mixing 2 μL of 1 mM YOYO-1 with 50 μg of DNA followed by an incubation for 2 h in the dark at room temperature. All DNA:PEI polyplexes were prepared in 5% of the initial culture volume with a total of 1 μg DNA/mL of culture to be transfected. An equal volume of diluted PEI was added to the DNA. The mixture was inverting and incubated for 10 min at room temperature before its addition to cell culture.

2.4. Secreted alkaline phosphatase activity measurement

Determination of SEAP activity was performed as previously described (Durocher et al., 2000). Briefly, SEAP was assessed at 6 days post-transfection (dpt). SEAP-transfected cells were centrifuged and the supernatant was diluted in water (1/1000). Fifty microliters of diluted supernatant were transferred to a 96-well plate and mixed with an equal volume of SEAP assay solution containing 20 mM paranitrophenyl phosphate (pNPP), 1 mM $MgCl_2$, and 1 M diethanolamine. Absorbance was read at 405 nm at 1-min intervals for 5 min.

2.5. Antibodies production

Cells grown in 2 L-shaker flasks to a density of 2.0×10^6 cells/mL were co-transfected with the *Trastuzumab*, *Palivizumab or B72-3* LC and HC plasmids at a LC:HC ratios of 1:1, 1:1 or 3:2 (w:w), respectively. A 54-mL suspension of polyplexes was incubated for 10 min before being added to the cultures. Twenty-four hours later, the cultures were fed with Tryptone N1 at 0.5% w/v final for 293-6E cells or 1% w/v final for CHO-3E7. One mL culture samples were collected daily for 8 dpt. Antibody titers were determined by protein-A HPLC using a 800 μL POROS® 20 μm Protein-A ID Cartridge (Applied BioSystems, Foster City, CA, USA) according to the manufacturer's instructions. The protein-A HPLC method showed less than 5% inter-assay variation and was validated using a calibrated USP standard (Synagis®).

2.6. Quantification of N-propionyl residues

We quantified the amount of N-propionyl residues in each polymer backbone as their abundance influences the number of protonatable amines and thus, may have significant impact on polyplex formation and transgene expression. Prior to analysis, PEI"Max" and LPEI were diluted in a mixture of 90% phosphate-buffered saline and 10% D_2O at a concentration of 4.5 mg/mL. For PEIpro™, a 1 mg/mL stock solution was concentrated 5 times using Amicon Ultra-15 (3 K) centrifugal filter cartridge (Millipore) and diluted in D_2O at a concentration of 4.5 mg/mL. NMR experiments were performed at 298 K on a Bruker Avance 800 NMR spectrometer equipped with a 5 mm z-gradient PATXI probe. One-dimensional (1D) ^{1}H NMR spectra were acquired with 512 scans, 4 dummy scans and a spectral width of 20.0332 ppm using 32 k data points. Water suppression was achieved using excitation sculpting

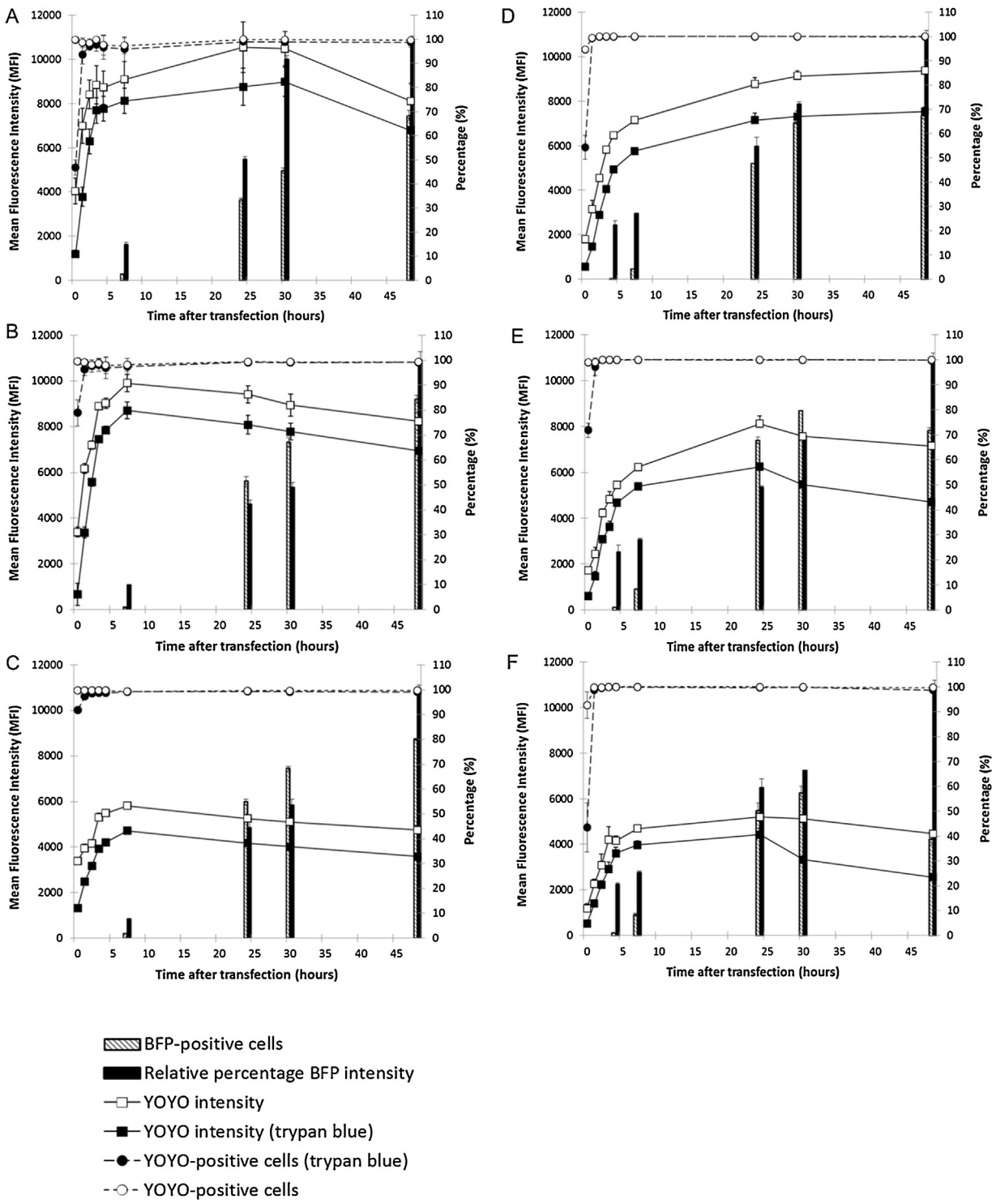

Fig. 3. Uptake kinetics of PEI-based polyplexes and blue fluorescent protein expression in HEK293 and CHO cells. HEK293 (A–C) and CHO (D–F) were transfected with LPEI (A and D), PEI"Max" (B and E) and PEIpro™ (C and F). Polyplexes were formed with optimal DNA:PEI weight ratios (Table 2). Prior to complexation, DNA were labeled with YOYO-1. The DNA/YOYO-1 mean fluorescence intensity associated with the cells (empty squares), the percentage of cells with bound polyplexes (empty circles), the mean fluorescence intensity of internalized polyplexes (filled squares) and percentage of cells with internalized polyplexes (filled circles) were quantified by flow cytometry. BFP was used as reporter protein to determine transfection efficiencies. The BFP mean fluorescence intensity (dashed bars) and percentage of BFP-positive cells (black bars) were also measured by flow cytometry at indicated times post-transfection. Data are an average of three independent experiments.

with gradients (Hwang and Shaka, 1995). The multiplicity edited two-dimensional (2D) ^{1}H–^{13}C HSQC (*Heteronuclear Single Quantum Coherence*) experiment (Willker et al., 1993) was collected as a 512 × 64 complex data matrix with spectral widths of 10.0166 ppm in the ^{1}H dimension and 80 ppm in the ^{13}C dimension, and 128 scans for each increment. All NMR spectra were processed with the Bruker TopSpin™ 3.0 software. Prior to Fourier Transformation, the 1D ^{1}H time domain data were zero-filled to 64k data points and an exponential line broadening factor of 1 Hz was applied. The 2D time domain data at the ^{13}C dimension was extended by linear predic-

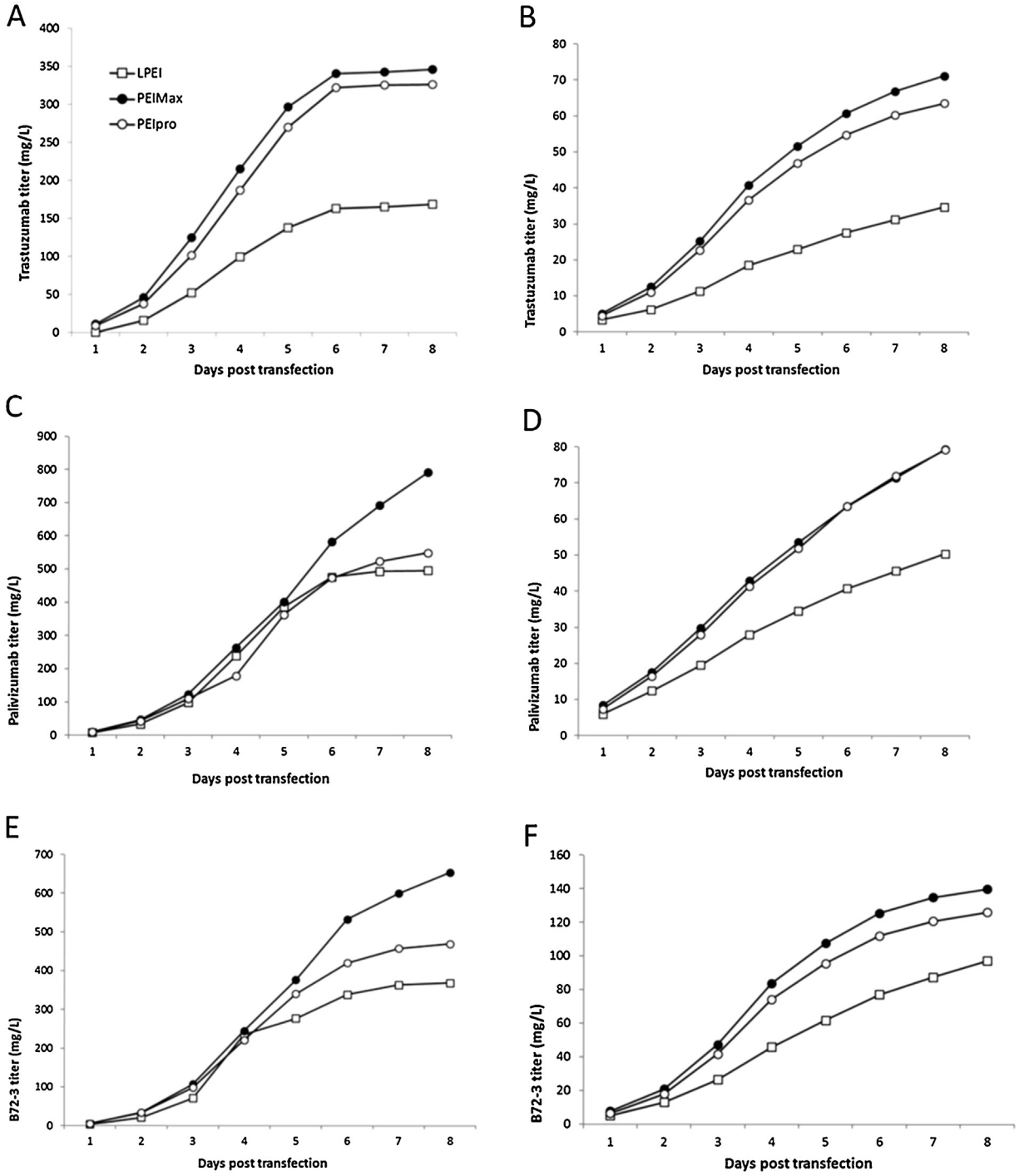

Fig. 4. Antibody expression following transfection with PEIs. Five hundred milliliters of HEK293 (A, C, E) and CHO (B, D, F) cell cultures were transfected with LPEI (empty squares), PEI"Max" (filled circles) and PEIpro™ (empty circles) using optimal DNA:PEI weight ratios for each polymer (Table 2). The cultures were sampled daily up to 8 days post-transfection. Titers of the monoclonal antibodies *Trastuzumab* (A, B), *Palivizumab* (C and D) and *B72-3* (E and F) were determined by protein-A HPLC.

tion followed by zero filling obtaining the data size of 2048 × 512 along the ^{1}H and ^{13}C dimensions, respectively. A $\pi/2$ shifted cosine window functions were applied in both ^{1}H and ^{13}C dimensions.

2.7. Flow cytometry analysis

Polyplexes attachment to the cell surface, internalization and transgene expression kinetics were performed at 10 min, 1, 2, 3.5, 4.5, 7, 24, 30 and 48 h post-transfection (hpt). Two hundred microliters aliquots of transfected cell culture were taken from the 6-well plates and filtered through a 310 μm mesh Nitex tissue (Sefar Group). Fluorescence signals of YOYO-1 labeled DNA were recorded on a BD LSR II flow cytometer (BD Biosciences). Then, a 0.4% (w/v) trypan blue solution was added to a final concentration of 0.2% to quench cell surface-associated fluorescence, thus allowing for DNA internalization quantification. Data obtained were mean fluorescence units of 10,000 single viable cells. To assess integrity of the cell membrane, 2 μL of a 1 mg/mL propidium iodide (PI) solution in water were added to each tube containing 200 μL of transfected cell culture. Cells were vigorously mixed before analysis. Viable

Table 2
Optimal DNA:PEI ratios (w:w) and equivalent amine (N in PEI) to phosphate (P in DNA) ratios leading to the most efficient SEAP expression.

	293-6E		CHO-3E7	
	w:w	N:P	w:w	N:P
LPEI	1:3	23:1	1:8	61:1
PEI"Max"	1:3	13:1	1:5	21:1
PEIpro™	1:1	4:1	1:2,5	11:1

cell density was determined by flow cytometry using a dot plot of forward light scatter versus 90° side scatter.

3. Results

3.1. Optimization of DNA:PEI ratios for HEK293 and CHO cells transfection

We first established the DNA:PEI ratio leading to maximal gene expression in 293-6E and CHO-3E7 cells for LPEI, PEI"Max" (referred as PEImax hereafter) and PEIpro™. Culture media are known to significantly impact transfection efficiencies due to the presence of various components such as anti-aggregation polymers and chelating agents (Eberhardy et al., 2009; Li et al., 2011). To avoid discrepancies in data interpretation resulting from the use of different culture media, both 293-6E and CHO-3E7 cells were adapted to the same protein-free, chemically-defined F17 medium and transiently transfected with a pTT plasmid encoding SEAP. The optimal DNA concentration of 1 μg per mL of cell culture and the incubation time (10 min) that allowed for proper complex formation were previously determined in our group (data not shown).

Fig. 1A displays the productivities obtained following transfection of 293-6E cells. We can observe an overall bell-shaped profile of the SEAP expression level as a function of the DNA:PEI ratio employed. As a decline in SEAP expression concomitant with a drastic decrease in viability were observed, a maximal concentration of 3 μg and 4 μg PEIpro™ per μg DNA was used to transfect HEK293 and CHO cells. The optimal ratio was found to be 1:3 (w:w) for LPEI and PEImax, and between 1:1 to 1:1.5 for PEIpro™. Similarly, the optimal DNA:PEI ratios were determined for CHO-3E7 cells (Fig. 1B). At 6 dpt, the highest level of SEAP obtained with PEIpro™ was achieved at a DNA:PEI ratio of 1:3 while it was obtained with ratios of 1:5 and 1:8 for PEImax and LPEI, respectively. These optimal ratios to transfect 293-6E and CHO-3E7 cells are summarized in Table 2.

3.2. Cytotoxicity of PEIs-based polyplexes

As previously reported (Carpentier et al., 2007; Raymond et al., 2011), increasing the quantity of PEI beyond a certain level results in decreased productivity due to increased cytotoxicity. The overall system performance is thus the result of an ideal balance between transfection efficacy and cytotoxicity. Therefore, we investigated the cytotoxicity of each PEI-based polyplexes at 48 hpt using the PI fluorescent dye exclusion assay. The mean intensity of PI-positive cells after transfection (dashed lines) was determined by flow cytometry and represented in Fig. 1C (293-6E) and 1D (CHO-3E7). We observed an increase in PI incorporation with increasing DNA:PEI ratios for both cell lines. These data suggest a significant change in plasma membrane integrity and perhaps membrane function once the cells come into contact with polyplexes and/or free PEIs. Losses in cell membrane integrity were correlated with a decreased 293-6E and CHO-3E7 cell viability (solid lines) as shown in Fig. 1 (panels C and D, respectively). Cell culture viability was assessed by the light scattered by the cells during flow cytometry analysis. Viability decreased with increasing quantity of PEIs. We found that PEIpro™-based polyplexes were the most toxic for both cell lines, reducing the viability of 293-6E and CHO-3E7 cells by 42% and 18%, respectively, at a concentration of 3 μg/mL. It was correlated with the fastest increase in PI intensity compared to that of LPEI- and PEImax-based polyplexes. However, at the optimum DNA:PEI ratios for maximal SEAP productivity, cell cytotoxicity remained low and were comparable for all polymers (viable cell density 93% ± 0.1 for LPEI, 91% ± 0.3 for PEImax and 92% ± 0.9 for PEIpro™).

3.3. Acylation level of polyethylenimines

The multiplicity edited $^{1}H–^{13}C$ HSQC (*Heteronuclear Single Quantum Coherence*) experiment was performed to assign the CH, CH_2 and CH_3 groups in the PEIs backbone. We determined the abundance of methyl ($–CH_3$) groups in the three PEI solutions as a measure of acylation levels. As PEImax and PEIpro™ are claimed to be extensively deacylated compared to LPEI (according to both manufacturers), a significant reduction in the methyl group signal is expected. The 1D ^{1}H NMR spectrum of each PEI is shown in Fig. 2. The signals centered at 3.20, 2.95 and 3.00 ppm (peak a) could be assigned as the methylene (–CH2–) protons adjacent to amide groups in the ethylenimine unit. The triplet peaks (d) centered at 1 ppm originated from the methyl protons of N-propionyl ethylenimine unit. The successful removal of N-propionyl chains in the PEImax and PEIpro™ was confirmed by the loss of the peaks b and d that are well visible in the LPEI spectrum. From the 1D ^{1}H NMR spectra, the CH_3:CH_2 ratios were determined by calculating the relative area of methylene proton peaks in the ethylenimine backbone to the methyl signal of the N-propionyl ethylenimine units. As expected, the data suggest that LPEI exhibits more N-propionyl groups with a CH_3:CH_2 ratio of 2.01:100, while it is reduced to 0.16:100 and 0.35:100 for PEImax and PEIpro™ respectively. Thus, PEImax and PEIpro™ bear respectively 12- and 6-times less propionyl groups compared to LPEI.

3.4. Attachment and endocytosis kinetic of polyplexes

We evaluated the attachment and internalization kinetics of polyplexes by flow cytometry in 293-6E (Fig. 3, panels A–C) and CHO-3E7 (Fig. 3, panels D–F) cells to examine whether binding and/or uptake of polyplexes differ between PEI types and cell lines. In this experiment, the optimal DNA:PEI ratio determined previously was used (Table 2). Polyplexes were prepared with PEIs and YOYO-1 labeled pTT-BFP vector. Although YOYO-1 intercalates into DNA before polyplexes were formed, it has been shown that YOYO-1 labeling does not affect DNA packaging (Burke and Pun, 2008) nor the transgene expression (our observation, data not shown). A first measure of fluorescence issued from cell surface-bound and internalized YOYO-1 labeled DNA was achieved by flow cytometry. This measure was referred to as polyplexes "attachment". A second measure was performed following quenching extracellular fluorescence with the membrane-impermeant dye trypan blue (Loike and Silverstein, 1983). This measures internalized polyplexes and is referred to as polyplexes "endocytosis".

With 293-6E cells, a very fast attachment of LPEI-, PEImax- and PEIpro™-based polyplexes was detected at 10 mpt with more than 99% of HEK293 cells being YOYO-1-positive (empty circles of Fig. 3, panels A–C respectively). However, flow cytometry analysis in the presence of trypan blue showed that internalization kinetic was polymer-dependent, with polyplexes made with PEIpro™ being the most rapidly internalized by 293-6E cells, followed by those made with PEImax and finally LPEI, with 91.8%, 78.9% and 46.8% of cells showing internalized polyplexes at 10 mpt (closed circles of panels A–C respectively). Even though all 293-6E cells had

internalized polyplexes after 1 hpt (closed circles), the overall capture/internalization of fluorescent polyplexes per cell still increased rapidly until 7 hpt (empty and black squares of panels A–C). Contrary to PEImax and PEIpro™ polyplexes, LPEI-based polyplexes were still taken up by 293-6E cells after 7 hpt until 30 hpt (black squares of Fig. 3A). Interestingly, the mean fluorescence intensity analysis showed that the amount of PEIpro™-based polyplexes bound and internalized by 293-6E cells was half that of LPEI and PEImax.

For CHO-3E7 cells, a rapid and efficient binding of polyplexes was also measured at 10 mpt, with more than 99% of cells being YOYO-positive when using LPEI, PEImax and PEIpro™ (empty circles of Fig. 3 panels D–F respectively). Flow cytometry analysis in the presence of trypan blue showed that internalization kinetic was also polymer-dependent, but this time PEImax was the most rapidly internalized followed by LPEI and finally PEIpro™, with respectively 71.9%, 54.3% and 43.5% of cells showing internalized polyplexes at 10 mpt (closed circles of panels E, D and F, respectively). As observed with 293-6E cells, all CHO-3E7 cells had internalized polyplexes at 1 hpt (closed circles) and the maximal amount of internalized PEIpro™-based polyplexes was approximately half of that observed with LPEI and PEImax polyplexes. The time needed to reach the maximal amount of attached (empty squares) and endocytosed (black squares) polyplexes by CHO-3E7 cells was longer compared to 293-6E cells. Maximum mean fluorescence intensities were reached at 24 hpt for PEImax- and PEIpro™-based polyplexes (panels E and F) whereas it still slightly increased after 24 hpt for LPEI-based polyplexes.

3.5. Blue fluorescent protein expression

Polyplex binding and internalization is merely the first step for an efficient TGE. Once internalized, the DNA has to reach the nucleus in order to be efficiently transcribed. Blue fluorescent protein (BFP) expression was monitored to verify and compare the efficacy of the three PEIs for TGE. The time needed for detectable BFP gene expression (closed bars), despite the small percentage of positive cells (hatched bars), was comparable for all PEIs tested, being of 7 hpt for 293-6E and 4.5 hpt for CHO-3E7 cells (Fig. 3). The transfection efficiencies given by the percentage of BFP-positive cells were still increasing at 48 hpt for 293-6E cells while it peaked at 30 hpt for CHO-3E7 cells. Our transfection protocol leads to an efficient BFP expression in both cell types using the three different PEIs. In fact, 68–84% of 293-6E and 58–80% of CHO-3E7 cells expressed the transgene.

3.6. Antibody productions

Finally, we compared the effectiveness of the three PEIs for the expression of three different mAbs. Expression of *Trastuzumab* (Fig. 4A and B), *Palivizumab* (Fig. 4C and D) and the chimeric *B72-3* (Fig. 4E and F) mAbs was performed by co-transfection of plasmid DNA encoding the light (LC) and heavy (HC) chains. The optimal DNA:PEI ratios determined previously (Table 2) were used to transfect 500 mL cell cultures in 2 L-shaker flasks. Samples were collected daily and antibody titers were measured by protein-A HPLC. Productions were harvested 8 dpt when the viabilities were 40–70% (data not shown). The measured antibody titers were greater in both cell lines when using PEImax and PEIpro™ compared to LPEI. For HEK293 cells (panels A, C, E), transfections using PEImax-based polyplexes lead to highest expression for all antibodies eventhough PEIpro™ resulted in yields only slightly lower for the *Trastuzumab* antibody. For CHO cells (panels B, D, F), the use of PEImax and PEIpro™-based polyplexes consistently resulted in highest antibody titers. The maximum *Trastuzumab, Palivizumab* and chimeric *B72-3* antibody titers obtained when using PEImax were respectively 346, 791 and 653 mg/L for 293-6E cells and 71, 79 and 140 mg/L for CHO-3E7 cells. Growth curves and percentage of viabilities related to each antibody production are presented in Supplemental Fig. 1.

4. Discussion

The increasing need for the fast production of quantities of recombinant proteins (r-proteins) has led to the development of large-scale TGE in animal cells cultivated in serum-free suspension cultures. Although PEIs are efficient synthetic gene delivery vehicles, their efficacy (expression level obtained per gene copy delivered) is much lower than that of viral vectors (Wang et al., 2013; Yin et al., 2014). Chemical modifications of PEIs have been performed to enhance one or more events involved during polyplex-mediated gene delivery, such as binding to the cell surface, entry into the cells *via* endocytosis, escape from the endosomal compartment, translocation to the nucleus and polyplex unpacking (Fortier et al., 2014; Thomas and Klibanov, 2002). In the present study, we compared three commercially available linear PEIs, LPEI, PEImax and PEIpro™, for their ability to transfect HEK293 and CHO cell lines. We first optimized the DNA:PEI ratios for each cell line in order to find the one supporting the highest volumetric productivity using the reporter protein SEAP. For each cell line and PEI used, this condition represents the “sweet spot” defined by the compromise between transfection efficiency and cell viability. As previously described, the quantity of internalized DNA and the level of transgene mRNA production can be increased beyond the maximal volumetric productivity condition by increasing the amount of polyplexes on a per cell basis, but this does not result in improved transgene expression, due to reduced cell growth and/or viabilities associated with PEI cytotoxicity (Carpentier et al., 2007). Our ^{1}H NMR analysis indicated that LPEI polymer is 97.9% deacylated (i.e. 2.1% of proprionyl group), consistent with previous data (Benjaminsen et al., 2013), while PEImax and PEIpro™ polymers are 99.8% and 99.6% deacylated, respectively. Increasing the number of protonatable amine residues may improve endosomal escape of polyplexes via the “proton sponge effect” and consequently, increase the transfection efficacy (Boussif et al., 1995; Cho et al., 2003; Thomas et al., 2005). We could also observe from the major NMR methylene chemical shifts distribution that the PEIpro™ polymer is more uniform than LPEI and PEImax polymers. This observation is confirmed by SEC analysis (see Fig. 1 in (Delafosse et al., 2016)) whereby the elution profile of PEIpro™ was more homogeneous than that of LPEI and PEImax, indicating that the latter are significantly more polydisperse. The peak b observed on LPEI 1D ^{1}H NMR spectrum corresponds to the methylene protons in the ethylenimine unit that are adjacent to N-propionyl groups. As expected, the vanishing of this peak in PEImax and PEIpro™ ^{1}H NMR profiles was correlated with the elimination of N-propionyl chains. Even though our results confirmed that PEImax and PEIpro™ are more deacylated than LPEI, the additional number of protonatable amines seems minor. According to our NMR experiment and assuming a molecular weight of 25 kDa, there is 19.25 and 21.46 additional protonatable amines per mole of PEIpro™ and PEImax respectively. We wanted to know if this difference, as small as it is, could significantly affect physico-chemical characteristics of our polyplexes and thus r-protein production yields. Not surprisingly, the gel retardation assay experiment (see Fig. 2 in (Delafosse et al., 2016)) showed that deacylated PEIpro™ and PEImax did not significantly increase their DNA condensation ability. Interestingly, when polyplexes were formed at optimal DNA:PEI ratios, size distribution and zeta potential measurements (Supplemental Table 1) showed that all three PEIs could condense DNA into particles of small sizes (Z-average between 66 and 96 nm) compatible with

efficient endocytosis (Garaiova et al., 2012) and comparable zeta potentials (22–32 mV). However, when optimizing the DNA:PEI ratios to adequately transfect HEK293 and CHO cells, we found that the fraction of unbound, or free PEI, is specific to each polymer and cell line. For instance, we observed that 0.6, 2.5 and 2.5 μg excess of PEIpro™, PEImax and LPEI respectively are needed per μg of DNA to optimally transfect HEK293 cells whereas this amount is increased to 2.1, 4.5 and 7.5 μg respectively for CHO cells. No correlation between the degree of deacylation and the optimal quantity of PEI was observed. Perhaps polydispersity and average size of a polymer are more important physico-chemical characteristics for the formation of TGE-competent nanoparticles. Indeed, efficient transfection required less free PEIpro™ which exhibits a higher molecular weight and lower polydispersity in comparison with LPEI and PEImax. On the other hand, LPEI and PEImax displayed similar apparent molecular weight and polydispersity, their acylation level being the main measurable difference between the two polymers.

The efficacy of a polymer on the transfection process is commonly based on end-point reporter gene expression levels. We presented here data providing information related to intracellular events such as polyplex attachment to the cell surface and endocytosis. We did not investigate precisely the endocytic pathway involved in the two cell lines but according to the data presented herein, polyplex attachment and endocytosis are not limiting factors under our transfection conditions. Indeed, all of the cells had internalized polyplexes at 1 hpt, indicating that internalization is a rapid and efficient process. Moreover, polyplexes were able to bind more than 98% of the cells within 10 mpt. Polyplexes with higher positive charge might improve cell uptake by enhancing their electrostatic interactions with the negatively charged glycosaminoglycans present at the cell surface. We did not see a correlation between polyplexes internalization kinetics and their zeta potentials. In fact, polyplexes formed with PEIpro™ at a DNA:PEI ratio of 1:3 (w:w) and LPEI at a ratio of 1:8 exhibit identical zeta potential values. Interestingly, while the maximal amount of internalized PEIpro™-based polyplexes was approximately half of that observed with LPEI and PEImax polyplexes, comparable percentages of BFP-expressing cells were observed. This indicates that the intracellular events following endocytosis that lead to gene expression are more efficient using PEIpro™. It has to be noted that our transfection conditions result in efficient BFP expression in both cell types using the three different PEIs even though they behave differently in terms of “attachment” and “endocytosis”. In fact, at least 58% and as much as 84% of HEK293 and CHO cells expressed the BFP transgene. We finally chose three mAbs of interest to be expressed in HEK293 and CHO cells. Despite similar amounts of internalized polyplexes, those made with PEImax resulted in significantly higher mAb productivity (155–230%) compared to LPEI. Noticeably, while endocytosis levels of PEIpro™-based polyplexes were half of that obtained with LPEI (and PEImax), the mAb expression levels were also always higher with PEIpro™ compared to LPEI (110–213%). Overall, CHO and HEK293 cells transfected with PEImax- and PEIpro™-based polyplexes gave better production yields (with a benefit for PEImax-based polyplexes in HEK293 cells) compared to LPEI. Furthermore, PEImax is more cost-effective compared to PEIpro™.

An additional level of complexity is the variable capacity of one particular PEI to mediate transfection of different cell types (Midoux et al., 2008; von Gersdorff et al., 2006). One would expect that cells with lower plasmid uptake activity would demonstrate lower level of gene expression. Monitoring polyplexes binding and internalization showed that, for each PEI, the amount of internalized polyplexes was very similar between HEK293 and CHO cells for a given polymer. However, under identical experimental conditions (except for the DNA:PEI ratio), HEK293 cells exhibited 4- to 10-fold higher mAb production yields than CHO cells. Comparison of intrinsic transfectability and productivity of each cell line is complicated by the existence of multiple variables. For example, the CMV promoter present in the pTT vectors, although relatively strong in both cell types, is much stronger in HEK293 cells and consequently higher transgene expression levels can be obtained (Gilbert et al., 2007). Also, significant improvement in CHO transient gene expression can be achieved through the addition of valproic acid combined with AKT co-transfection and a temperature shift to 32 °C 24 hpt (Durocher and Loignon, 2009; Wulhfard et al., 2010). Under these conditions, productivities of 200 mg/L for *Trastuzumab* and 280 mg/L for *B72-3* were obtained (data not shown). Additionally, we noticed that the kinetics by which polyplexes enter cells may be an important parameter leading to efficient gene transfer, an observation in accordance with previous results obtained in our laboratory (Paris et al., 2008). It is possible that the delayed endocytosis of polyplexes observed in CHO cells is somehow linked to the internalization pathway(s) leading to their cytoplasmic sequestration, thus limiting transfection efficacy (Thibault et al., 2010).

5. Conclusion

Both HEK293 and CHO cell lines are widely used for the transient production of research-grade r-proteins. Besides, the generation of stable CHO cell lines is still often used as TGE does not always meet the required productivities. Optimizing the transfection process to allow for higher r-protein productivities is thus desirable. The proper selection of the transfection reagent represents an easy parameter to optimize and may have significant impact on TGE process. PEImax and, to a lesser extent, PEIpro™ are more deacylated than LPEI according our ^{1}H NMR analysis. The use of PEImax or PEIpro™ polymers to transiently transfect HEK293 and CHO cells clearly provide increased productivities compared to LPEI. Since size-exclusion chromatography experiment reveals that PEImax and LPEI exhibit the same apparent molecular size, deacylation may be responsible for the enhanced transfection efficiency observed with PEImax. As for PEIpro™, its higher apparent molecular weight and/or increased monodispersity, combined with its low acylation level, may explain why this polymer is more efficient (similar productivities compared to PEImax but with half of internalized polyplexes). When using optimal DNA:PEI ratios, the resulting polyplexes are positively charged and their size range (<100 nm) allows for proper endocytosis. Kinetics of polyplex-attachment/internalization and transgene expression indicate that all HEK293 and CHO cells bind polyplexes within 10 mpt and internalized them within 1 hpt. Despite a robust BFP transgene expression was observed for all PEIs, not all cells express the reporter gene after 48 hpt indicating that downstream events limit transfection efficiency. Overall, PEImax and PEIpro™ appears to be the most effective polymers for TGE in HEK293 and CHO cells as confirmed by the productivity obtained for three monoclonal antibodies. Although these findings demonstrate the importance of determining the most suitable polymers to efficiently transfect CHO and HEK293 cells, further improvements of TGE would require the elucidation of the specific receptor(s)/coreceptors(s)/internalization pathway(s) that leads to an efficient DNA delivery into their nucleus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.04.028.

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